EFFECT OF NOVEL ERβ AGONIST IN CONTROLLING NEUROINFLAMMATION AND APOPTOSIS IN CEREBRAL ISCHEMIA IN RAT MODEL

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CERTIFICATE

This is to certify that the dissertation work entitled "**Effect of novel ERβ agonist in controlling neuroinflammation and apoptosis in cerebral ischemia in rat model"** submitted by **University Reg. No.261525901** is a bonafide work carried out by the candidate under the guidance of **Dr. M. Ramanathan, M. Pharm., PhD.,** and submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology** at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2016-2017.

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DECLARATION

I do hereby declare that the dissertation work entitled "**Effect of novel ERβ agonist in controlling neuroinflammation and apoptosis in cerebral ischemia in rat model"** submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology**, was done by me under the guidance of **Dr. M. Ramanathan, M. Pharm., PhD.,** at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2016-2017**.**

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EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled "**Effect of novel ERβ agonist in controlling neuroinflammation and apoptosis in cerebral ischemia in rat model"** submitted by **University Reg. No 261525901** to the Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology** is a bonafide work carried out by the candidate at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore and was evaluated by us during the Oct 2017.

Examination Center: PSG College of Pharmacy, Coimbatore.

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Dedicated

To

Respectful Guide,

Beloved Parents

&

God

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Abbreviations

ABBREVIATIONS

MCAo	**:**	**Middle cerebral artery occlusion**
BBB	**:**	**Blood brain barrier**
TGF -β	**:**	**Transforming growth factor**
nNOS	**:**	**Neuron nitric oxide synthase**
TNFR	**:**	**Tumour necrotic factor receptor**
CBF	**:**	**Cerebral blood flow**
CREB	**:**	**CAMP receptor element binding protein**
ATP	**:**	**Adenosine tri-phosphate**
eNos	**:**	**Endothelial-nitric oxide synthase3**
AD	**:**	**Alzheimer disease**
PD	**:**	**Parkinson disease**
ERK	**:**	**Extracellular signal-regulated kinases**
NO	**:**	**Nitric oxide**
MCP-1	**:**	**Monocyte chemo attractant protein-1**
IL-1α	**:**	**Interleukin-1alpha**
IL-1β	**:**	**Interleukin-1beta**
TNF-α	**:**	**Tumour necrosis factor-alpha**
iNOS	**:**	**Inducible nitric oxide synthase**
EER	**:**	**Estrogen regulated receptor**
PR	**:**	**Progesterone receptor**
AR	**:**	**Androgen receptor**
ER	**:**	**Estrogen receptor**
SR	**:**	**Steroid receptor**

ELISA	**:**	**Enzyme linked immunosorbent assay**
CBP	**:**	**CREB binding protein**
CAMK	**:**	**Calcium calmodium dependent protein kinase**
LOX	**:**	**Lipoxygenase**
IL-6	**:**	**Interleukin-6**
CCL2	**:**	**Chemokines ligand 2**
MIP-1	**:**	**Macrophage inflammatory protein 1**
ICAM	**:**	**Intracellular adhesion molecule**
MMPS	**:**	**Matrix metalloproteinases**
IGF-1	**:**	**Insulin like growth factor 1**
OVX	**:**	**Ovariectomized**
HRT	**:**	**Hormone replacement therapy**
SERM	**:**	**Selective estrogen receptor modulator**
LDL	**:**	**Low density lipoprotein**
ERKo	**:**	**Estrogen receptor knockout**
αERKo	**:**	**Estrogen receptor alpha knockout**
βERKo	**:**	**Estrogen receptor beta knockout**
RUTH	**:**	**Raloxifene use for the heart**
SNP	**:**	**Single nucleotide polymorphism**
HERS	**:**	**Heart and estrogen progestine replacement study**
EPM	**:**	**Elevated plus maze**
IAEC	**:**	**Institutional animal ethical committee**
IR	**:**	**Ischemic reperfusion**
ANOVA	**:**	**Analysis of variance**
CCA	**:**	**Common carotid artery**

ECA	**:**	**External carotid artery**
ICA	**:**	**Internal carotid artery**
CNS	**:**	**Central nervous system**
CPCSEA	**:**	**Committee for the purpose of control and supervision of experiments of animals**
NF-κB	**:**	**Nuclear factor kappa-light-chain-enhancer of activated B cell**

Introduction

1. INTRODUCTION

Estrogen receptors (ERs) belong to the steroid hormone superfamily of nuclear receptors (NRs) (Katzenellenbogen *et al*.,1996). Other types of steroid receptors among the NRs include the estrogen-related receptors (EER), progesterone receptors (PR), androgen receptors (AR), glucocorticoid, and mineral corticoid receptors. Steroid receptors (SRs), act as ligand-dependent transcription factors, and their activity is associated with the cell cycle (Lee *et al*.,2012; Kim *et al*.,2012). Estrogens, acting via the ER, play important roles in regulating the growth, differentiation, and functioning of many reproductive tissues including the uterus, vagina, ovary, oviduct, and mammary gland. In the uterus and mammary gland, estrogens increase proliferation and alter cell properties via, at least in part, the induction of growth factors and growth factor receptors, an effect largely antagonized by antiestrogens (Katzenellenbogen *et al*.,1996). Moreover, estrogen is an important sex hormone produced primarily in the ovaries in females and testes in males. This steroid hormone regulates the growth, development, and physiology of the reproductive system in humans.

Estrogens have important sites of action in the pituitary, hypothalamus, and specific brain regions (Katzenellenbogen *et al*.,1996). Estrogen also affects the neuroendocrine, skeletal, adipogenic, and cardiovascular systems (Lee *et al*.,2012; Kim *et al*.,2012,). ERs are found mainly in the nucleus, but also in the cytoplasm and mitochondria (Jia *et al*.,2015; Wright *et al*.,2015). Estrogens have been shown to protect against neurodegenerative diseases and injury, including stroke (Westberry *et al*.,2008).Biological functions of estrogen are mediated by binding to the ERs estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Estrogen signaling is selectively stimulated or inhibited depending upon a balance between ERα and ERβ activities in target organs. ERα was cloned from human breast cancer MCF-7 cells in 1986 and ERβ was cloned from rat prostate in 1996 (Lee *et al*.,2012; Kim *et al*.,2012,). ERα and ERβ genes are located on different chromosomes 6q25.1 and 14q23.2 (Jia *et al*.,2015; Wright *et al*.,2015).

The proposed functions of ERβ include antiproliferative action, regulation of apoptosis, control of antioxidant gene expression, and modulation of immune responses, anxiety related behavior, and the risk of heart failure. In many respects, ERβ acts as an indispensable hormone receptor for maintenance of the proper functions of vital organs (Leung *et al.,2006*).

ER activates intracellular signaling pathways that indirectly affect genomic activity via other transcriptional regulators such as cyclic-AMP response element binding protein (CREB). CREB is abundantly expressed in the brain, particularly in neurons. Phosphorylation at the ser-133 position of CREB followed by recruitment of CREB binding protein (CBP) activates CRE-mediated gene transcription. Various extracellular stimuli cause phosphorylation of CREB (pCREB) via Protein Kinase A, extracellular signalrelated kinase (ERK), and calcium–calmodulin dependent protein kinase (CaMK). Antiinflammatory and neuroprotective activity of estradiol, neuroprotection after ischemia, inhibition of microglial activation, and inhibition of high MMP-9 levels. In addition, a growing number of studies have reported antiinflammatory roles for ERβ, such as neuroprotection after ischemia, inhibition of microglial activity, suppression of transcription, and decreased levels of IL-1b, IL-6, TNFα, and chemokine CCL2 (Asl *et al*.,2013; Khaksari *et al*.,2013)**.**

ERβ agonists in mice demonstrated that neuroprotection following global ischemia is mediated by ERβ (Carswell *et al*., 2004). More recently ERβ agonists were shown to have no effect following focal ischemia in rats (Farr *et al*., 2007). The role of ERβ could be due to the type of ischemia or differences between rats and mice. Studies using ERα knockout females, however, demonstrate that neuroprotection by estradiol following focal ischemia is dependent on the presence of ERα in the cortex (Dubal *et al*., 2001), and that ERβ alone is not sufficient for neuroprotection in females.

In the present study, the neuroprotective effect of novel ERβ agonists will be evaluated in middle cerebral artery occlusion model. To explore the behavioural alteration: grip strength, open field test, elevated plus maze will be performed. The neuroinflammation will be assessed by quantifying TNFα and IL1β (ELISA). The anti apoptotic activity will be evaluated by caspase activity assay and quantification of Bax/Bcl2 level (Western blot).

Literature Review

2.LITERATURE REVIEW

Estrogen Receptor

Estrogen receptors (ERs) belong to the steroid hormone superfamily of nuclear receptors (NRs) (Katzenellenbogen *et al*.,1996). Other types of steroid receptors among the NRs include the estrogen-related receptors (EER), progesterone receptors (PR), androgen receptors (AR), glucocorticoid, and mineral corticoid receptors. Steroid receptors (SRs), act as ligand-dependent transcription factors, and their activity is associated with the cell cycle (Lee *et al*.,2012; Kim *et al*.,2012). Estrogens, acting via the ER, play important roles in regulating the growth, differentiation, and functioning of many reproductive tissues including the uterus, vagina, ovary, oviduct, and mammary gland. In the uterus and mammary gland, estrogens increase proliferation and alter cell properties via, at least in part, the induction of growth factors and growth factor receptors, an effect largely antagonized by antiestrogens (Katzenellenbogen *et al*.,1996). Moreover, estrogen is an important sex hormone produced primarily in the ovaries in females and testes in males. This steroid hormone regulates the growth, development, and physiology of the reproductive system in humans. Estrogens have important sites of action in the pituitary, hypothalamus, and specific brain regions (Katzenellenbogen *et al*.,1996). Estrogen also affects the neuroendocrine, skeletal, adipogenic, and cardiovascular systems (Lee *et al*.,2012; Kim *et al*.,2012). ERs are found mainly in the nucleus, but also in the cytoplasm and mitochondria (Jia *et al*.,2015; Wright *et al*.,2015). Estrogens have been shown to protect against neurodegenerative diseases and injury, including stroke (Westberry *et al*.,2008).

ER Subtypes

Biological functions of estrogen are mediated by binding to the ERs estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Estrogen signaling is selectively stimulated or inhibited depending upon a balance between ERα and ERβ activities in target organs. ERα was cloned from human breast cancer MCF-7 cells in 1986 and ERβ was cloned from rat prostate in 1996 (Lee *et al*.,2012; Kim *et al*.,2012). ERα and ERβ genes are located on different chromosomes 6q25.1 and 14q23.2 (Jia *et al*.,2015; Wright *et al*.,2015).

ERβ belongs to the nuclear receptor superfamily. It is classified as a type I nuclear receptor, because it resides in cytosol and undergoes nuclear translocation after ligand binding. ERβ is expressed in a variety of normal and malignant tissues, some of which express ERα .The proposed functions of ERβ include antiproliferative action, regulation of apoptosis, control of antioxidant gene expression, and modulation of immune responses, anxiety related behavior, and the risk of heart failure. In many respects, ERβ acts as an indispensable hormone receptor for maintenance of the proper functions of vital organs (Leung *et al.,2006*).

Different between ERα and ERβ

ERα and ERβ contain different ligand binding domains, and each receptor is encoded by a different gene. This receptors also signal differently at the AF-1 site in the presence of Estrogen, the Estrogen activates transcription at ERα, it inhibits transcription at ERβ, respectively. ERα and ERβ receptors have been implicated to mediate genomic signaling as well as non-genomic signaling in cells. Another important difference between ERα and ERβ is tissue distribution, ERα is expressed in breast, ovary, uterus, and brain, while ERβ is expressed in bone, heart, lungs, kidney, endothelial cells and brain. In the brain,ERα is localized most densely in the hypothalamus, hippocampus, and preoptic area, with moderate to light density in the cerebral cortex. ERβ localization has been documented predominantly in the cortex, throughout the hippocampus, in the olfactory bulb, septum, preoptic area, nucleus of striata terminalis, amygdala, paraventricular hypothalamus, thalamus, ventral tegmental area, substantia nigra and cerebellum (Brann *et al*.,2012; Raz *et al*.,2012).

ROLE OF ER IN ISCHEMIC STROKE

Estrogens are steroid hormone known to play a crucial role in the cellular events involved in development and maintenance of normal body function throughout a lifetime by acting on a vast variety of target tissues(Morissette *et al*.,2008; Saux *et al*.,2008). Estrogen has potent neuroprotective effects in animal models of brain disease or injury via various mechanisms such as anti-inflammation, reduction of oxidative stress and excitotoxicity, anti-apoptosis and vasodilation in the ischemic brain.

Estrogens have been shown to protect against neurodegenerative diseases and injury, including stroke. Permanent middle cerebral artery occlusion (MCAo) is a wellestablished model of focal ischemic stroke in rodents. Following MCAo, there is a reduction in cerebral blood flow, which leads to cell death in the striatum followed by damage to cells in the overlying cortex (Namura *et al*., 1998; Liu *et al*., 1999). In this model, gonadectomized females (Simpkins *et al*., 1997; Dubal *et al*., 1998) and males (Toung *et al*., 1998; Alkayed *et al*., 1998) have a much larger MCAo-induced cell loss than animals with higher circulating estrogen concentrations. Pre-treatment with even low doses of 17-β estradiol is sufficient to exert dramatic protection in the brains of both female (Dubal *et al*., 1998; Dubal *et al*., 2001) and male rats (Toung *et al*., 1998). Many of estrogen's actions occur via two nuclear receptors, estrogen receptor alpha (ERα) (Koike *et al*.,1987) and the more recently discovered estrogen receptor beta (ERβ) (Kuiper *et al*.,1996). These receptors are important for neuroprotection by estrogen. Generalized pharmacologic blockade of estrogen receptors exacerbates ischemic injury in mice (Sawada *et al*.,2000) and blocks estrogen-induced neuroprotection in cultured neurons (Singer *et al*., 1999; Wilson *et al*.,2000) and in cortical explant cultures (Wilson *et al*.,2000). Another study using ERα knockout mice also suggested that in male ERα knockout mice, the absence of ERα did not increase the ischemia-induced damage, but these males were not given estradiol (Sampei *et al*., 2000). In both male and female rodents, ERα mRNA expression is high in the neonatal cortex, but dramatically decreased to only a few cells in the uninjured adult cortex. Twentyfour hours after MCAO, however, ERα mRNA and protein are significantly increased in the cortex of female rats and mice (Dubal *et al*., 1999, 2006). In ovariectomized (OVX) females, this increase in ERα expression occurs in both oil and estradiol-treated groups, but is seen earlier after injury with estradiol treatment (Dubal *et al*., 2006). These data suggest that in females, the ischemia-induced increase in ERα expression in the cortex is necessary for the neuroprotective effects of estradiol. In males, the injury induced regulation of ERα and the mechanisms of estradiol action are still largely unknown. ERβ agonists in mice demonstrated that neuroprotection following global ischemia is mediated by ERβ (Carswell *et al*., 2004). More recently ERβ agonists were shown to have no effect following focal ischemia in rats. The role of ERβ could be due to the type of ischemia or differences between rats and mice. Studies using ERα knockout females, however, demonstrate that neuroprotection by estradiol following focal ischemia is dependent on the presence of ERα in the cortex (Dubal *et al*., 2001), and that ERβ alone is not sufficient for neuroprotection in females.

STROKE

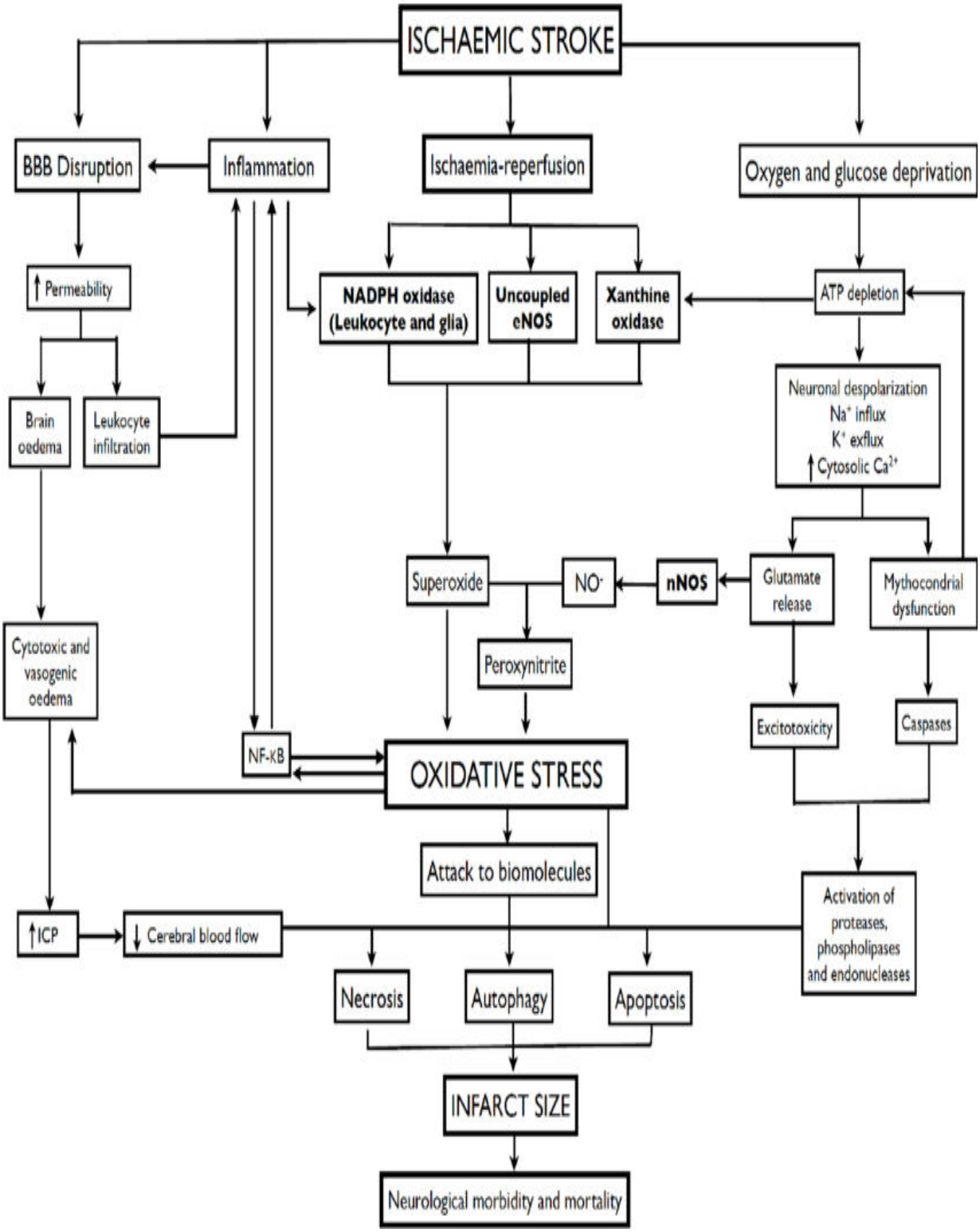
The two broad categories of stroke, hemorrhage and ischemia,they are diametrically opposite conditions. Hemorrhage is characterized by too much blood within the closed cranial cavity, while ischemia occurs when the amount of oxygen and other nutrients supplied by blood flow is insufficient to meet the metabolic demands of brain tissue (Tuttolomondo *et al*.,2012; Raimondo *et al*.,2012). such as glucose,critical for survival of central nervous system (CNS) cells (Tobin *et al*.,2014; Bonds *et al*.,2014).

Ischemic Stroke

The most common type of stroke is called ischemic stroke or cerebral infarction. According to recent data released by the American Heart Association, 87 % of strokes are classified as ischemic(Roger *et al*.,2012; Go *et al*.,2012). Infarcts occur as a result of insufficient or interrupted flow of blood to an area of the brain, typically caused by blockage of an artery . Brain tissue must be supplied with oxygen, glucose, and other vital materials by the constant influx of blood at a rate of about 50–54 ml of blood per 100 g of brain tissue per minute. Brain cells deprived of adequate blood flow (cerebral blood flow [CBF] below 15–20 ml blood flow/100g tissue/min) will become ischemic(Astrup *et al*.,1981; Symon *et al*.,1981) their membrane pumps will begin to fail, intracellular processes will break down, and the brain tissue becomes swollen. Importantly, the ischemic brain tissue may still be salvageable if perfusion can be restored at this point . If the hypoperfusion worsens (i.e., CBF <8–10 ml/100 g/min) (Hossmann *et al*.,1994), this tissue at risk will become irreversibly damaged with cell death proceeding within 4–8 min. This event is referred to as an ischemic stroke . Damage to brain tissue will occur initially in the region contiguous to the blocked artery (the so-called infarct core), while a penumbra of at-risk tissue surrounding this region remains viable for some time following onset of ictus and with timely intervention may respond to efforts to restore blood flow to the region(Astrup *et al*.,1981; Symon *et al*.,1981).

MECHANISM OF ISCHEMIC STROKE

FIGURE .1.



TYPES OF ISCHEMIA

Focal ischemia:Focal ischemic stroke occurs when cerebral blood flow is attenuated in a specific brain region (Hata *et al*.,2000; Maeda *et al*.,2000).Dependent on the nature of the occlusion in the cerebral artery, focal ischemic stroke can be further subdivided into thrombotic or embolic stroke(Adams *et al*.,1993; Amarenco *et al*.,2009).

Thrombotic stroke occurs when a blood clot is formed within a cerebral artery, which is commonly caused by atherosclerosis where the vascular endothelium is constantly damaged resulting in the activation of numerous vasoactive enzymes that leads to the formation of an atherosclerotic plaque within the cerebral artery. Furthermore, additional pathological changes in atherosclerosis such as thrombosis, ulceration and calcification increases the risk of blood clot formation. Other pathological conditions such as hypercoagulable states, fibromuscular dysplasia, arteritis and arterial trauma can comparably lead to thrombotic strokes (Broussalis *et al*.,2012; Killer *et al*.,2012).

Embolic strokes occurs when a blood clot or atherosclerotic plaque fragment that is formed elsewhere in the circulatory system detaches and is mobilized through the blood stream and occludes a cerebral artery**.** The most common artery to be occluded by an embolus are the left and right middle cerebral arteries since 80% of blood volume that travels through the arteries in the neck eventually flows through the middle cerebral artery (Demaerschalk *et al*.,2010)

Global ischemia: Global ischemic stroke occurs when blood flow to the entire brain or a majority part of the brain is stopped or severely reduced due to hemodynamic changes in the peripheral circulatory system (Böttiger *et al*.,1999; Teschendorf *et al*.,1999).

Ischemic Stroke Models

Several focal cerebral ischemic stroke models have been developed in a variety of species; these include mechanical occlusion of the middle cerebral artery (MCA), thrombo embolic models, and photothrombotic models. Global ischemia models, although not formally stroke, are divided into complete and incomplete models of ischemia, which are produced by occluding the cerebral blood flow (CBF) completely or incompletely (Yan *et al*.,2015; Chopp *et al*.,2015).

Animal models of ischemic stroke (Yan *et al*.,2015; Chopp *et al*.,2015)

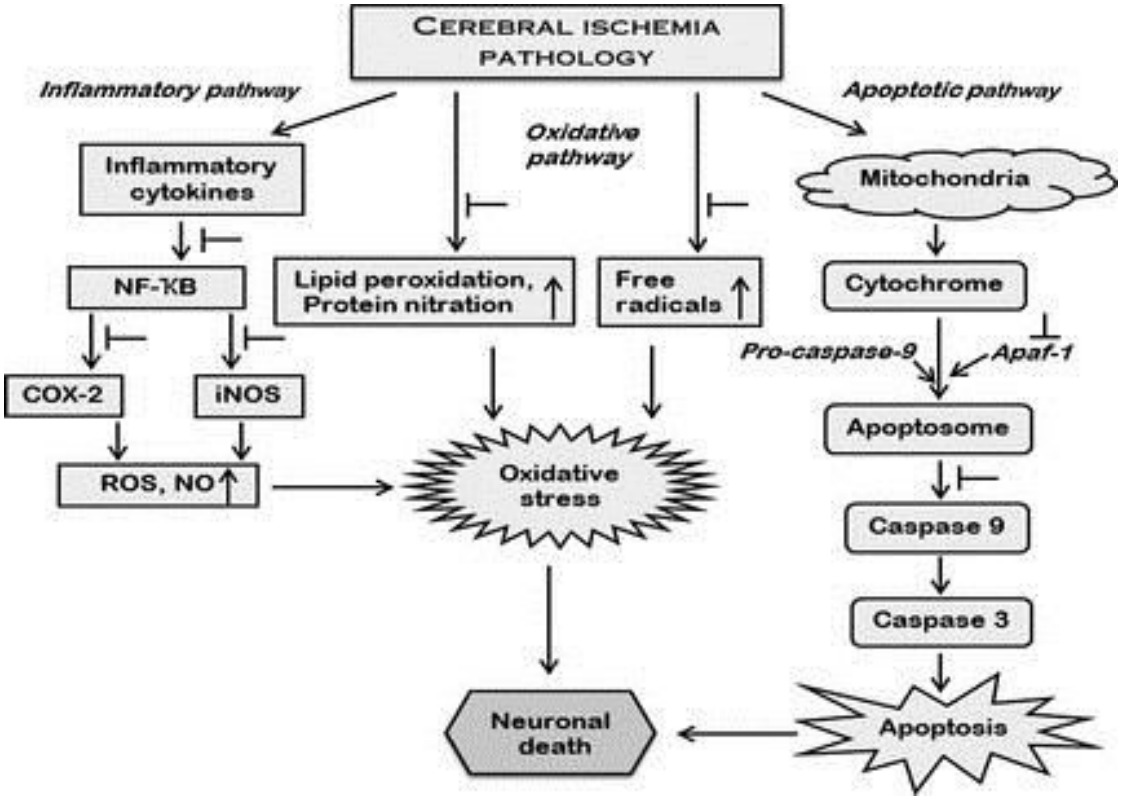
TABLE.1.

Types	Means of induction	Characteristics
Focal ischemic models		
Mechanical Mcao models		
Surgical clips	Clip proximal MCA	Technical feasibility and consistent histological results
MCA ligation	Ligate the MCA	Lesion limited to cortex
Eletrocoagulation	Interrupt MCA blood flow by electrocoagulation	Induces distal and proximal MCAO
Intraluminal filament MCAO	Insert filament to origin of MCA	Reproduces cerebral ischemia and reperfusion injury
Embolic		
Thrombotic	Occlude MCA by thrombotic clot made with autologous blood	Best model for study of thrombolytic agents
Microspheres	Inject calibrated compounds and artificial microspheres into MCA	Different sizes of emboli induce different ischemic events
Photothrombotic	Inject photosensitive dye	Facilitates behavioral studies of ischemia in specific anatomical-functional regions.
Endothelin-1	Apply ET-1 to exposed MCA	Induces dose-dependent reduction in local blood flow

Global ischemic models		
4-VO	Ligate carotid artery and vertebral arteries	Induces reversible bilateral forebrain and brainstem Ischemia
2-VO	Ligate bilateral carotid arteries	Induces reversible forebrain ischemia
3-VO	Occlude common carotid and basilar arteries	Better control of CBF than 4-VO
Ventricular fibrillation	Induce cardiac arrest with ventricular fibrillation	To study mechanisms of CA-induced delayed neuronal death.
Neck tourniquet	Inflate high-pressure cuff to occlude bilateral	Leads to variable ischemic outcomes.

Pathology of ischemia

FIGURE .2.



Important inflammatory mediator in ischemic stroke

After brain injury, up regulated inflammation mediator and immune cell infiltrate in the pathophysiology of cerebral ischemia acts as a complex role. This includes a series of proinflammatory mediators and anti-inflammatory mediators; it has a different role in the occurrence and development of ischemic stroke (Jin *et al*.,2013; Lin *et al*.,2013; Zhang *et al*.,2013; Nanda *et al*.,2013).

Proinflammatory mediators

IL-1α and IL-1β, IL-1α and IL-1β knockout mice in the middle cerebral artery occlusion can reduce brain damage. The main function of IL-1α and IL-1β was achieved by IL-1R1 and IL-1R2 (Rothwell *et al*.,2000; Luheshi *et al*.,2000).

ICAM-1 is one of the members of the immunoglobulin super family is a major proinflammatory cytokines, in stroke-related research, animal experiments showed that increased expression of ICAM-1 is related to the pathogenesis of ischemic lesions , while blocking or knockout ICAM-1 will reduce brain damage and improve stroke outcomes. Clinical studies soluble ICAM-1 is up-regulated and reached a peak in acute ischemic stroke within 24 hours. ICAM-1 also significantly up regulated in brain tissue after cerebral ischemia, but the real role of ICAM-1 in stroke still remained controversial (Shyu *et al*.,1997; Chang *et al*.,1997; Lin *et al*.,1997).

Chemokines Monocyte chemo attractant protein-1 (MCP-1), macrophage inflammatory protein -1(MIP-1α) and chemokine (CX3CL1), these three are the most common proinflammatory chemokines, up regulated in cerebral ischemia animal models , ischemic chemokine up regulation is seen as deleterious effect . Related studies have shown that MCP-1, MIP-1α, and CX3CL1 promote stroke pathological process; inhibiting or defect itself can reduce ischemic brain damage. MMPs Previous studies showed that matrix metallo proteinases (MMPs) and neurogenic migration are related, inhibition of MMPs activity may reduce brain damage . In the later phase of stroke due to MMP-9 associated with many growth factors, which are closely related with angiogenesis, therefore it is also beneficial for stroke (Soriano *et al*.,2002; Amaravadi *et al*.,2002).

Anti-inflammatory mediators

IL-10 Interleukin-10 (IL-10) is an anti-inflammatory cytokine, increased in the brain tissue after a stroke, at the same time plays an important role in the pathogenesis of stroke. Related studies show that IL-10-deficient mice have wider range of brain damage in middle cerebral artery occlusion , in animal models, after increased IL-10, the level of brain damage was decreased after the stroke (Kim JS *et al*.,1996; Yoon SS *et al*.,1996) , clinical studies have shown that low levels of IL-10 would indicate increased risk of stroke (van *et al*.,2002; Gussekloo *et al*.,2002).

TGF-β mRNA increased within 6 hours after stroke, the increasing levels last for 15 days after the stroke (Klempt *et al*.,1992), transforming growth factor-β(TGF-β) has Anti-inflammatory and neuroprotective effect in stroke, which may become effective stroke treatment strategies. TGF-β can reduce ischemic damage and reduce the associated inflammation , TGF-β blockade will aggravate ischemic brain damage (Johnston *et al*.,2001; Carter *et al*.,2001; Freed *et al*.,2001).

TIPE2 (Tumor necrosis factor-a-induced protein 8-like 2) is an anti-inflammatory protein, has an important role in maintaining the steady-state within the immune system ,mice with knockout TIPE2 will induce multi-organ inflammation and splenomegaly, studies show that TIPE2 has high expression in cerebral ischemia, but also has a role in the pathogenesis of stroke, blocking TIPE2 in mice that with middle cerebral artery occlusion can increase infarct size, neurological disorders, inflammatory cytokine expression, and inflammatory cell permeability (Zhang *et al*.,2012; Wei *et al*.,2012; Liu *et al*.,2012; Liu *et al*.,2012).

IGF-1 In an animal model, Insulin-like growth factors-1(IGF-1) having a neuroprotective effect after a stroke and can reduce infarct size and improve cell survival studies show that high levels of IFG-1 in serum during the early stage of ischemic stroke is closely related to neurological recovery and better functional outcome (Jin *et al*.,2013; Liu *et al*.,2013; Zhang *et al*.,2013; Nanda *et al*.,2013).

Iinflammation regulation are closely related; NF-kB pathway is more complex, NF-kB promotes neuronal death in cerebral ischemia. In cerebral ischemia NF-kB expression up regulated, the activation of NF-kB will become the agonists of TNF-a, IL 6 (Hallenbeck *et al*.,2002). Inhibition of autophagy can reduce the activity of NF-kB pathway, blocking the formation of autophagy small bodies and inhibition of lysosomal proteases, will reduce IkBa and NF-kB target gene expression. Autophagy degradation and inhibiting the phosphorylation to regulate IKK-b activity. The similarity such as TNF-α, IL-1 β and other cytokines were regulated by autophagy and produced during the inflammatory process. Beclin1, Beclin2 interacting protein is a key component in PI3K, it is very crucial during the formation of autophgic bodies (Wang *et al*.,2002) . In Jiang Y's studies believe that tetracycline inhibit ischemic brain tissue autophagic activity, on this basis further to suppress the inflammatory process by reducing NF-kB pathways (Jiang *et al*.,2012; Zhu *et al*.,2012; Wu *et al*.,2012; Xu *et al*.,2012).

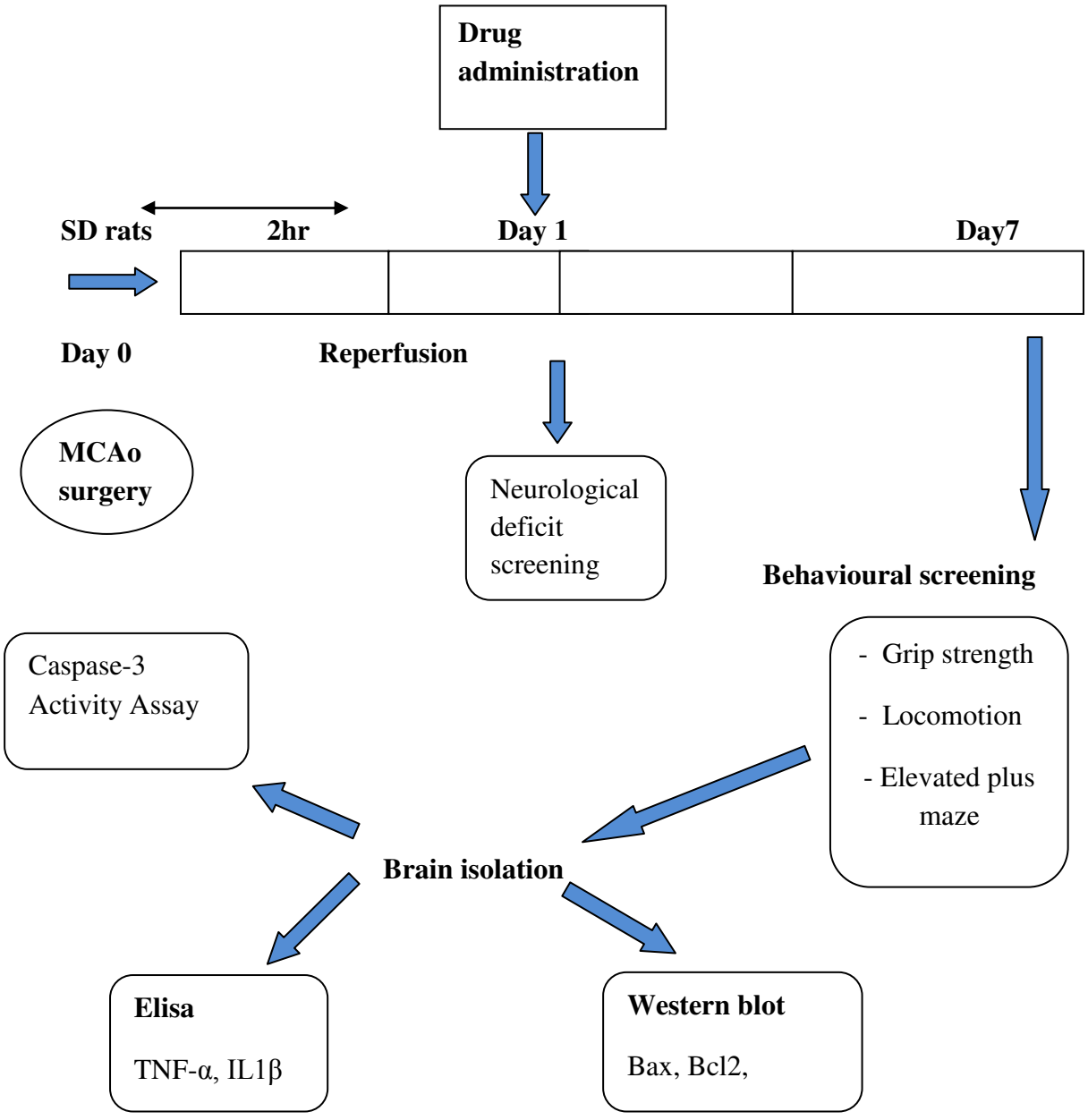
OBJECTIVE & PLAN OF STUDY

3. OBJECTIVES & PLAN OF STUDY

The objective of the study is

1. To assess behavioural and not studied functions of ER-β agonist in cerebral ischemic condition.
2. To evaluate the mechanism of ER-β agonist in cerebral ischemia by exploring the anti-inflammatory and apoptotic pathways.

PLAN OF STUDY



Phase-1

Surgical optimization and ischemia inducement by MCAo (middle cerebral artery occlusion) model in SD rats.

Phase-2

The following neurobehavioural parameter were studied to assess the effect of new leads in the MCAo model

2.1 Neurological deficit score (Zhang *et al*.,2016)

2.2 Gripstength (Leach *et al.,*1993*;* Esler *et al.,*1993)

2.3 Locomotor activity (Borlongan *et al.,*1995)

2.4 Elevated plus maze (EPM) (Vécsei *et al.,*1991; Beal *et al.,*1991)

Phase-3

The mechanism of action of neuroprotective action of ERβ agonist were evaluated by measuring the following biochemical markers through

3.1 Western blot

Bax, bcl2

3.2 Elisa

IL-1β,TNF-α.

3.3 Caspase-3 activity assay

Materials & Methods

4. MATERIALS & METHODS

MATERIALS:

TABLE.2.

Chemicals &tools used	Company name
Crushing forceps	Finetools
Bonney forceps	Finetools
Adson tissue forceps	Finetools
Adson teeth forceps	Finetools
Horton adson forceps	Finetools
Retractors	Finetools
Scalpels	Finetools
Microvessel clips	Finetools
Scissors	Finetools
Silicone gel	Anabond

Methods:

4.1. Animals

Male Sprague-Dawley rats (200-240g) used in the study were housed in individual polypropylene cages in a well ventilated room (air cycle: 15/hr) under an ambient temperature of 23±2ºC and 40-65% RH, with a 12:12 h light/dark artificial photoperiod. They were provided with food and purified water. All the animals were acclimatized atleast for 7days to the laboratory conditions prior to experimentation. Guidelines of "guide for the care and use of laboratory animals" were strictly followed throughout the study. Institutional animal ethical committee (IAEC), PSG Institute of medical science and research, Coimbatore, approved the study.

ER-β Agonist

FIGURE.3.

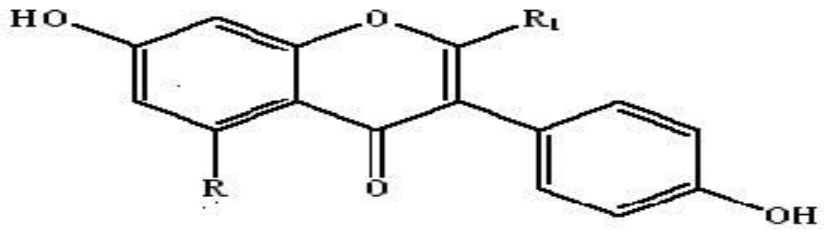


TABLE .3.

Compound	**R**	**R_1**
CMU	CH_3	-
CUE	-	C_2H_5

Groups and treatments :

TABLE.4.

Number	Group	Treatment
1	**CONTROL**	
2	**MCAO**	
3	**MCAO +**	**CMU 10mg/kg**
4	**MCAO +**	**CUE 10mg/kg**

4.2. Surgical procedure

Focal cerebral ischemia was induced by middle cerebral artery occlusion with minor modifications. Rats were anesthetized with ketamine (90mg/kg) and xylazine (15mg/kg).The left common carotid artery was exposed at the level of external and internal carotid artery bifurcation. 4-0 nylon monofilament was used and its tip was made round headed by exposing it to flame. The Filament was coated with Anabond silicone gel and inserted into the external carotid artery and advanced to the internal carotid artery for a length of about 20–21 mm until a slight resistance was felt. On achieving occlusion, the filament was held in place with ligature and the external incision was sutured temporarily. After 2 h of ischemia the rats were anesthetized, suture was opened, the filament was pulled out and reperfusion in internal carotid artery was ensured visually. Throughout the surgical procedure, body temperature was measured by inserting a thermometric probe into the rectum of rat and it was maintained at 37±0.5 °C using thermostatically controlled heating blanket. Animals were then kept in a cage with a heating lamp, which maintained the cage temperature between 29±1 °C for another 1 h to counteract any possible hypothermic effect. In the sham-operated (SO) group, external carotid artery was surgically prepared for insertion of filament, but the filament was not inserted.

4.3. Neurobehavioral paradigms

4.3.1. Neurological deficit (Zhang *et al*.,2016)

Neurological deficit were assessed by following score pattern after 24 hrs of ischemic reperfusion (IR)

Neurological deficits (6-point scale)

- 0 = No neurological deficit
- 1 = Failure to extend left forepaw fully
- 2 = Circling behaviour
- 3 = Falling to the right
- 4 = No spontaneous walking with a depression
- 5 = Death

4.3.2. Loco motor activity

The rats will be placed in the open field apparatus for recording the locomotion. The locomotor activity will be measured using an open field test. The behavioral parameters measured in open field test were Rearing, Grooming, No of ambulations, Time spent central compartment.

4.3.3. Grip strength measurement

Grip strength test by using bioseb grip strength meter was used to study of neuromuscular functions by determining maximal peak force developed by rodent were measured in units grams.

4.3.4 Elevated plus maze

The elevated plus maze test (EPM) consisted of two open arms (35 × 5 cm) crossed with two closed arms (35 × 5 ×20 cm). The arms were connected together with a central square of 5 × 5 cm. The apparatus was elevated to the height of 50 cm in a dimly illuminated room. Animals were placed individually at the end of either of the open arms facing away from the central platform. The time taken by each animal to move from open arm to either of the closed arms was recorded. This duration of time was called transfer latency (TL). If the

animal did not enter into any of the enclosed arms within 120 s, it was gently pushed into any of the enclosed arms and TL was considered as 120 s. Later the animal was allowed to explore the plus maze for 5 min and send back to its home cage.

4.4. Probable mechanism of neuroprotection

4.4.1 Western blot

Western Blot Analysis for Protein Detection Cells were lysed in RIPA buffer along with protease and phosphatise inhibitor cocktail. Samples were kept on ice for 15– 30 min followed by sonication and centrifugation at 12,000 rpm for 5 min. The supernatant solution was taken and assayed for total protein content by Bradford's method. Equal protein concentration (20 μg) was used for SDS polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane and blocked with 5 % bovine serum albumin prepared in TBST buffer for 3 h. The blocked membrane was incubated with primary antibody against Akt1 (1:750), S473 p-Akt1 (1:1000), GSK-3β (1:500), S9 p-GSK-3β (1:1000), β-catenin (1:500), and reference protein β-actin (1:1000) for 2 h at room temperature. Following that, goat anti-rabbit IgG-HRP and goat anti-mouse IgG1-HRP secondary antibodies were added to the membrane in separate procedures according to the primary antibody used in the previous step and incubated for 1 h at room temperature. The membrane was developed and detected by Gel Dock system, G: Box (Syngene, Frederick, MD, USA). Row volume was considered for analysis.

4.4.2. Elisa

The levels of TNF-α in the ischemic cortex tissue was detected using an enzyme-linked immune sorbent assay(ELISA) test as described previously (Hang *et al.,* 2004). Briefly, animals were killed 1 h, 4 h, and 24 h after MCAO. The brains were removed and cerebral cortex, dissected. The cortex was immediately frozen and kept at −70 °C. The frozen brain tissue was homogenized using a homogenizer in 500 μl buffer containing phosphate buffered saline (PBS) pH 7.2 (1mmol/L phenyl methyl sulfonyl fluoride (PMSF), 1 mg/L pepstatin A, 1 mg/Laprotinin, 1mg/L leupeptin) and centrifuged at 12,000g for20 min at 4 °C. Afterwards, supernatant was collected and total protein was determined by Micro BCA Protein Assay Kit. The level of TNF-α in ischemic cortex tissue supernatant was measured using an ELISA kit (Diaclone, France) specific for rat TNF-α. The measurement of TNF-α was performed step-

by-step based on the protocol booklet of ELISA kit. The TNF-α contents were expressed as pg TNF-α/mg total protein (Hang *et al.,* 2004).

4.4.3. Caspase activity assay

Brain tissue were harvested and lysed in 500 µl of lysis buffer by incubating on ice for 10 min. Centrifugation at 10,000×g for 2 min was done to remove cell debris. The supernatant extract was taken, and protein measurement was done by Bradford's method. Total protein (100 µg) was used in 50 µl of cell lysis buffer. To this, 50 µl of 2× reaction buffer containing 10 mMDTT and 5 µl of 4 mM of DEVD-pNA (caspase-3 substrate) was added and incubated at 37 °C for 90 min. Absorbance was measured at 405 nm by Multiskan™ GO multiplate reader(Thermo Scientific, Waltham, USA). The fold change in enzyme activity was measured.

4.5. Statistics

The data are expressed on mean ±SD. The results will be analysed through one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to assess the statistical significance of the differences among the study groups using Graph Pad Prism5.

Results

5. RESULTS

Phase 5.1: MCAo (Middle cerebral artery occlusion) surgery optimization

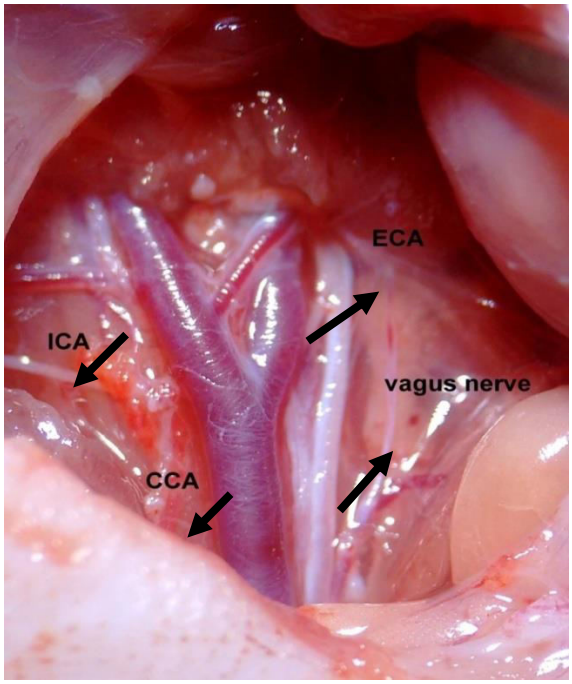


FIGURE.4. Branches of common carotid artery (CCA)

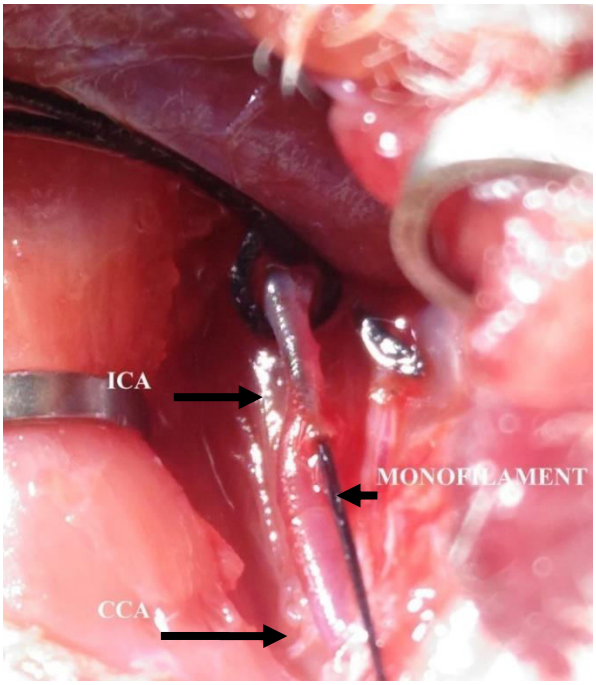


FIGURE.5. Insertion of the monofilament to ICA through ECA

Phase 5.2 Behavioural studies

5.2.1. Neurological deficit score

The neurological deficit in the MCAo rats was assessed after 24hrs IR and on 7th day after surgery. After 24hrs and on 7th day significant increase in neurological score in MCAo rats ($p<0.001$) was observed in comparison to control rats. Traumatic Treatment of CUE (10mg/kg), on 7th day showed significant decrease in neurological score in comparison to MCAo group.

5.2.2. Grip Strength

Data are expressed in force (gms). MCAo rats showed significant decrease ($p<0.001$) in muscle tone are evidently decrease in force applied on the grid when compared to vehicle treated rats. Treatment of NCE CMU (10mg/kg), CUE (10mg//kg) showed significant improvement in neurological score on noted by increase in force applied on grid comparison to MCAo rats.

5.2.3. Open Field Test

5.2.3.2. Effect on ambulations

MCAo rats showed significant decrease ($p<0.01$) in number of ambulations is comparison to control rats indicates loss of exploration behaviour. CUE (10mg/kg) showed significant increase $p<0.05$ in ambulatiory behaviour in comparison to MCAo group indicating improved exploration activity.

5.2.3.3. Effect on grooming & Central compartment exploration

There was no statistical difference between the groups in grooming behaviour and central compartment exploration activity between the treatment groups was observed in comparison to control rats.

5.2.3.4. Effect on rearing

MCAo rats showed significant decrease ($p<0.01$) in reaering activity when compared to control vehicle treated rats indicaties reduced exploration activity. There was no significant difference between MCAo and treatment groups was observed in rearing behaviour.

5.2.3.5. Effect on licking

MCAo rats showed significant decrease ($p<0.05$) in number of licking behaviour when compared to control rats. Other treatment groups licking behaviour remains unaltered.

5.2.3.6. Effect on freezing

MCAo rats exhibited significant increase ($p<0.001$) in freezing behaviour as observed by increase in freezing time when compared to control rats indicates depression. CMU (10mg/kg), CUE (10mg/kg) showed significant decrease in freezing time in comparison to MCAo rats.

5.2.4. Elevated plus maze

5.2.4.1. Effect on time spent in central compartment

Data are expressed in seconds. MCAo rats ($p<0.01$) showed significant decrease in central compartment activity when compared to control rats indicating increased anxiety in the rat. There was no significant difference between MCAo and treatment groups was recorded in this parameters.

5.2.4.2. Effect on number of entries in open arm & closed arm

The number of entries made in open & closed arm of elevated plus maze was found to be similar across all the groups studied.

5.2.4.4. Effect on time spent in open arm

MCAo rats have shown significant decreased ($p<0.001$) time spent in open arm when compared to control rats denotes increased anxiogenicity. CMU (10mg/kg), CUE (10mg/kg)

treated rats showed significant increase in time spent in open arm in comparison to MCAo group indicating the decrease in anxiety behaviour induced by ischemia.

5.2.4.5. Effect on time spent in closed arm

Data are expressed in seconds. MCAo rats ($p<0.001$) have spent more time in closed arm when compared to control rats. CMU (10mg/kg), CUE (10mg/kg) treatment resulted in significant decrease in time spent in closed arm in comparison to MCAo rats indicates attenuation of anxiety behaviour due to ischemia.

5.2.4.6. Effect on time to reach closed arm

Data are expressed in seconds. MCAo rats ($p<0.001$) showed significant decrease in time taken to reach closed arm in comparison to control rats indicates CMU (10mg/kg) ,CUE (10mg/kg) treatment reversed in this behaviour in comparison to MCAo rats.

5.3 Mechanism of action of neuroprotective action of ERβ agonist

5.3.1 Western blot

5.3.1.1. Effect of drug treatment on ratio of BAX level

Data are expressed in ratio. MCAo rats ($p<0.001$) demonstrated significant increase in BAX level when compared to control rats. CMU (10mg/kg), CUE (10mg/kg) treated rats had by reduced significant BAX level in comparison to MCAo rats.

5.3.1.2. Effect of drug treatment on ratio of Bcl2 level

Data are expressed in ratio. MCAo rats showed significant decrease ($p<0.05$) in Bcl2 level after ischemia in comparison to control rats. CUE (10mg/kg) treatment lead to significant increase in Bcl2 level when compared to MCAo group.

5.3.2. ELISA

5.3.2.1. Effect on pro-Inflammatory mediator(TNFα) by ELISA

Data are expressed in pg/mg protein. MCAo rats ($p<0.001$) inducement in SD rats significantly increase in TNFα levels when compared to control rats denotes increase in

inflammatory activity. CMU (10mg/kg), CUE (10mg/kg) treatment showed significant decrease in TNFα levels in comparison to MCAo vehicle treated rats indicating decrease in inflammatory markers release.

5.3.2.2. Effect on pro-inflammatory mediator (IL 1β) by ELISA

Data are expressed in pg/mg protein. MCAo rats have shown significant increase ($p<0.001$) in IL 1β level in comparison to vehicle treated control rats indicates increase in inflammatory activity during CMU (10mg/kg), CUE (10mg/kg) treated rats have shown significant decrease in IL 1β levels in comparison to MCAo rats indicaties alternation of inflammatory response with Erβ NCE administration.

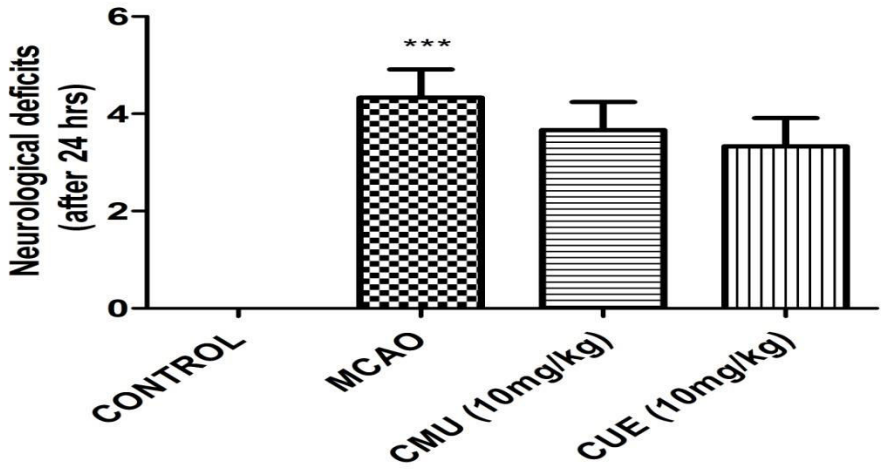
5.3.3. CASPASE-3 ACTIVITY ASSAY

5.3.3.1. Effect on caspase activity assay

Data are expressed in specific activity/µg protein. MCAo rats showed significant ($p<0.001$) increase in caspase enzyme activity in comparison to control rats shown increase in apoptosis (cell death). CMU (10mg/kg), CUE (10mg/kg) treatment resulted in cell proliferation significant decrease in caspase enzyme activity in comparison to MCAo rats indicating decreased observed by apoptosis.

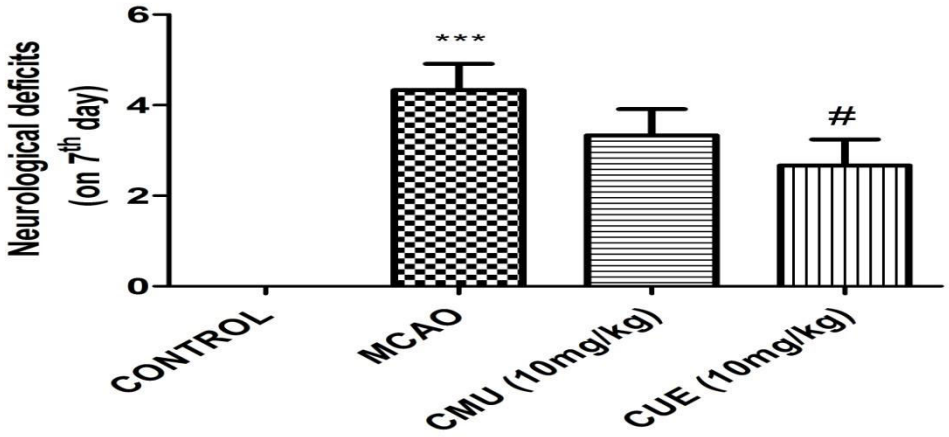
Behavioural Studies

FIGURE.6. Neurological deficit score after 24 hrs



Data are expressed as mean ±SD (n=6).Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. ***denotes statistical significance of MCAo group compared with control group at $p<0.001$.

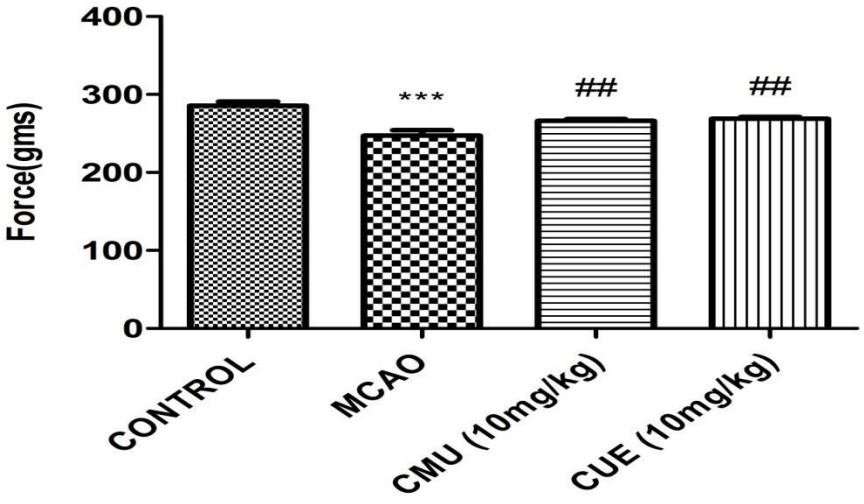
FIGURE.7. Neurological deficit score on 7th day



Data are expressed as mean ±SD (n=6). ***denotes statistical significance of MCAo group compared with control group at $p<0.001$. $^{\#}$denotes statistical significance of CUE (10mg/kg) treated groups compared with MCAo group at $p<0.05$ respectively.

Grip Strength

FIGURE.8. Effect of drug treatment on grip strength

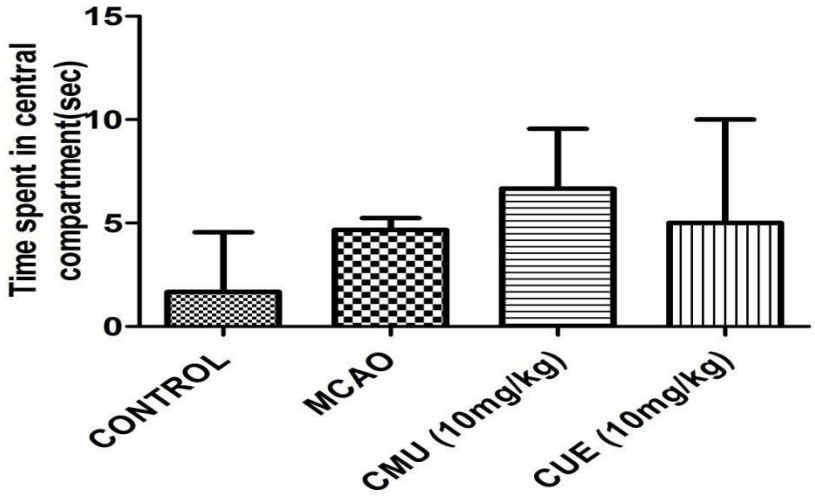


Data are expressed as mean ±SD (n=6). ***denotes statistical significance of MCAo group compared with control group at $p<0.001$. $^{\#\#, \#\#}$ denotes statistical significance of CMU

(10mg/kg), CUE (10mg//kg) treated groups compared with MCAo group at p<0.01, p<0.01 respectively.

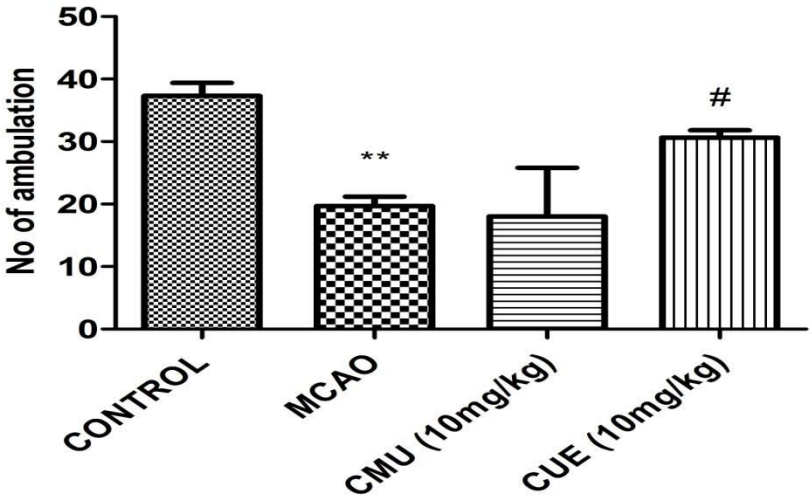
Open Field Test

FIGURE.9. Effect of drug treatment on time spent in central compartment



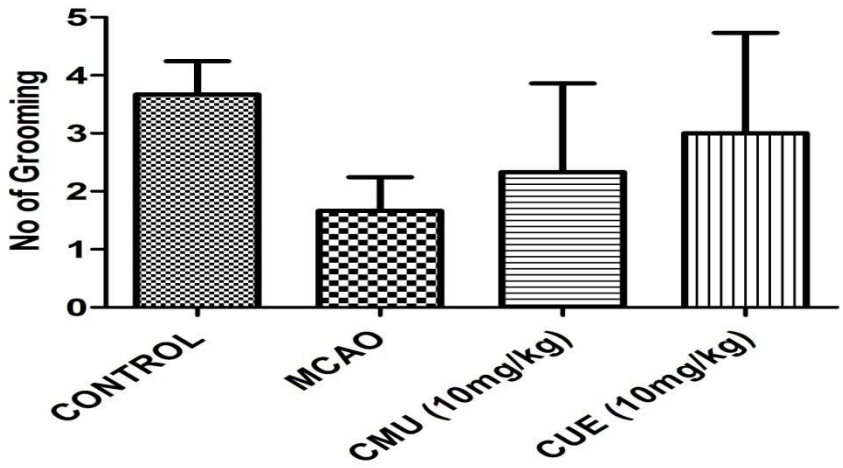
Data are expressed as mean ±SD (n=6).Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. There is no statistical significance between the groups.

FIGURE.10. Effect of drug treatment on no of ambulations



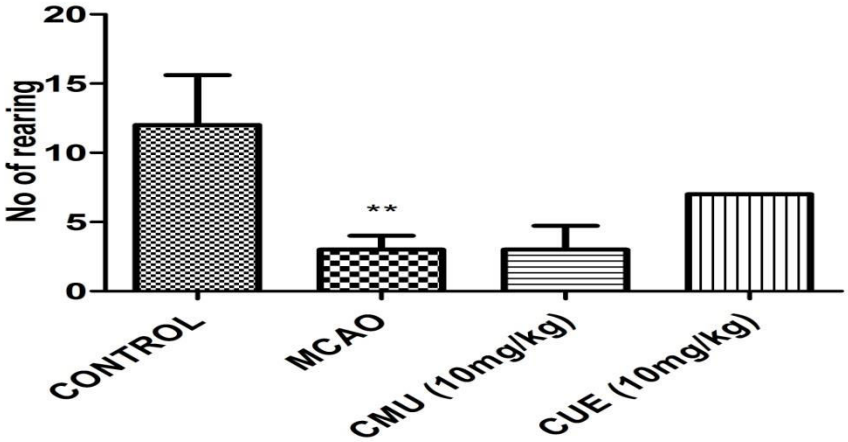
Data are expressed as mean ±SD (n=6). **denotes statistical significance of MCAo group compared with control group at p<0.01. #denotes statistical significance of CUE (10mg/kg) treated groups compared with MCAo group at p<0.05 respectively.

FIGURE.11. Effect of drug treatment on grooming



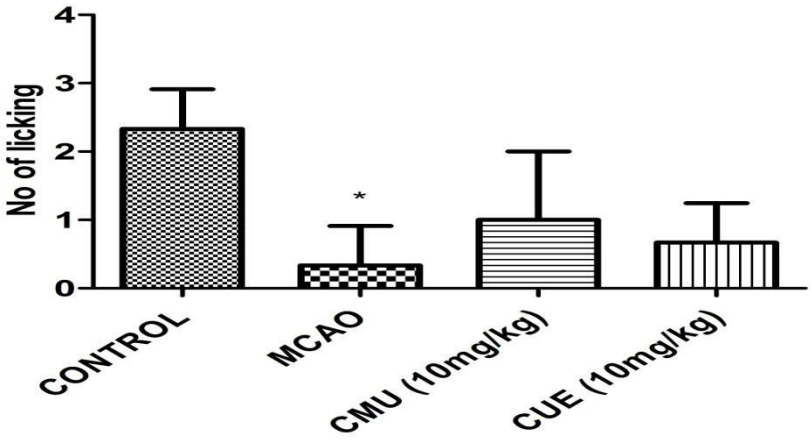
Data are expressed as mean ±SD (n=6).Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. There is no statistical significance between the groups.

FIGURE.12. Effect of drug treatment on rearing



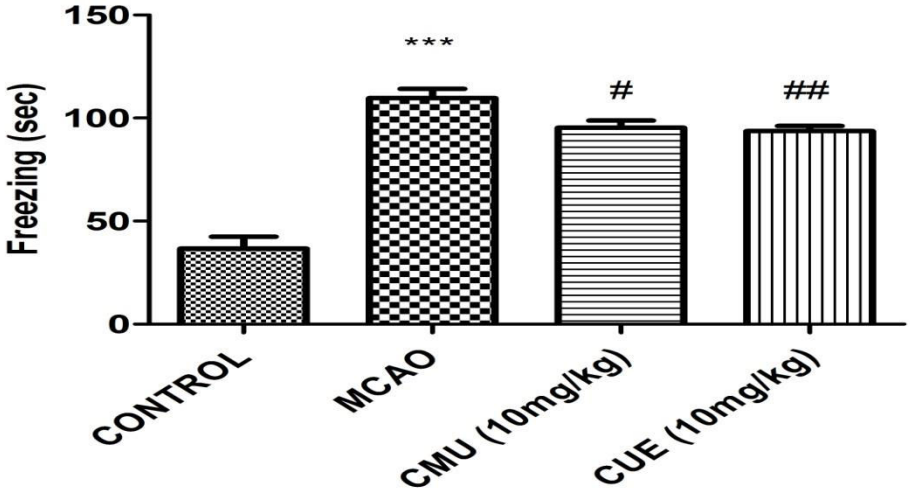
Data are expressed as mean ±SD (n=6).Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. **denotes statistical significance of MCAo group compared with control group at p<0.01.

FIGURE.13. Effect of drug treatment on licking



Data are expressed as mean ±SD (n=6).Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. * denotes statistical significance of MCAo group compared with control group at $p<0.05$.

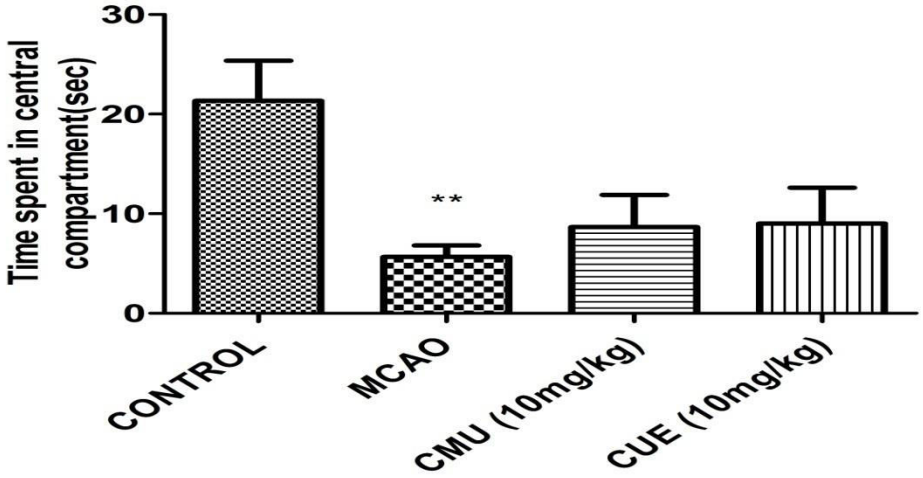
FIGURE.14. Effect of drug treatment on freezing



Data are expressed as mean ±SD (n=6).Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. *** denotes statistical significance of MCAo group compared with control group at $p<0.001$. #, ## denotes statistical significance of CMU (10mg/kg), CUE (10mg/kg) treated groups compared with MCAo group at $p<0.05$, $p<0.01$ respectively.

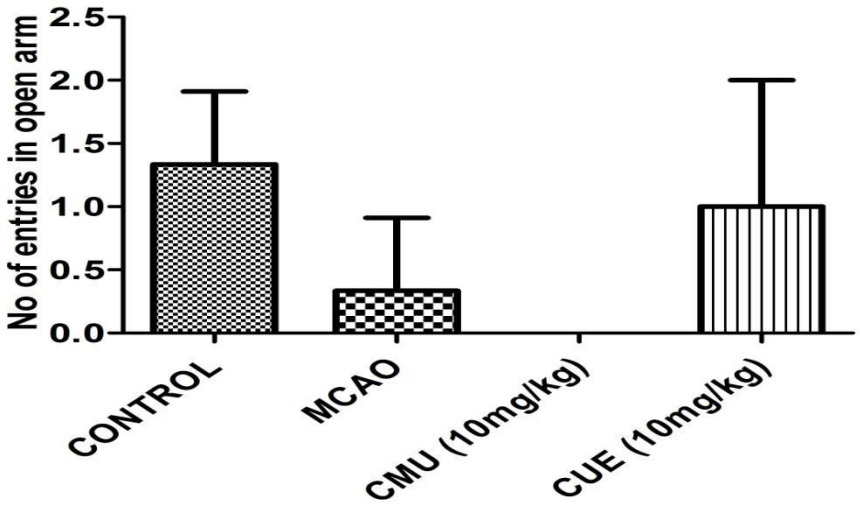
Elevated Plus Maze

FIGURE.15. Effect of drug treatment on time spent in central compartment



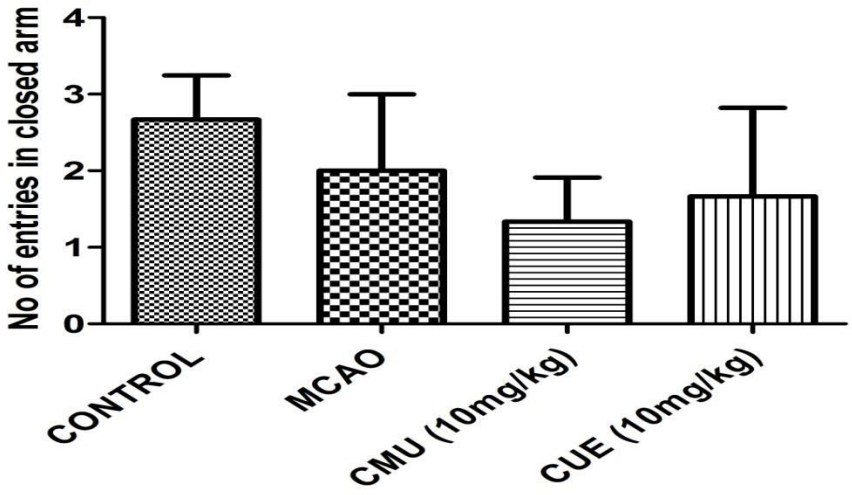
Data are expressed as mean ±SD (n=6). **denotes statistical significance of MCAo group compared with control group at $p<0.01$.

FIGURE.16. Effect of drug treatment on no of entries in open arm



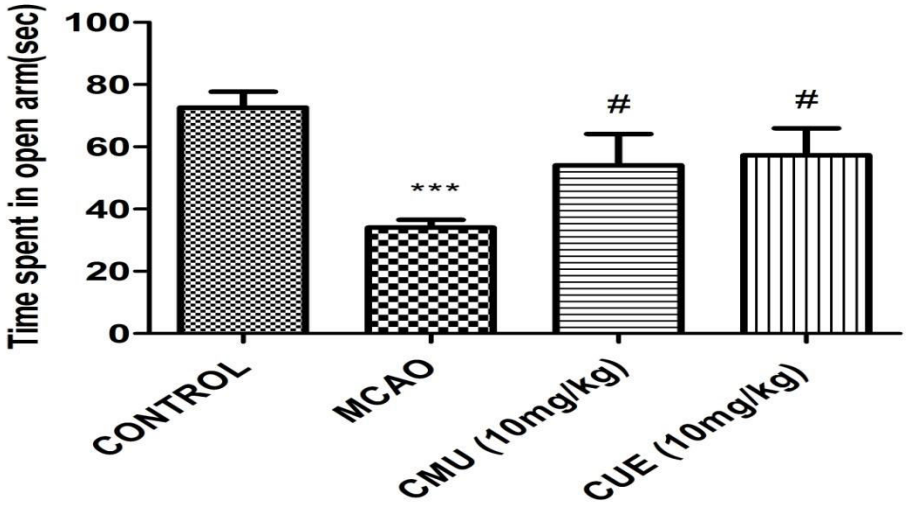
Data are expressed as mean ±SD (n=6).Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. There is no statistical significance between the groups.

FIGURE.17. Effect of drug treatment on no of entries in closed arm



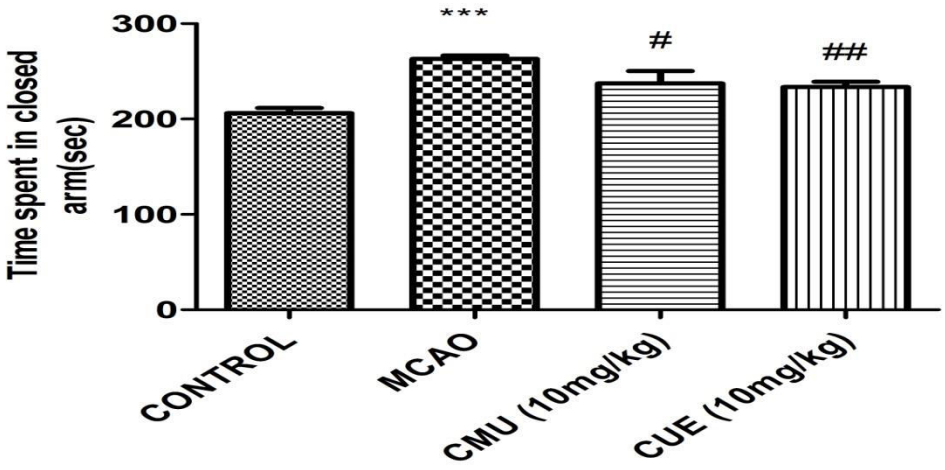
Data are expressed as mean ±SD (n=6). There is no statistical significance between the groups.

FIGURE.18. Effect of drug treatment on time spent in open arm



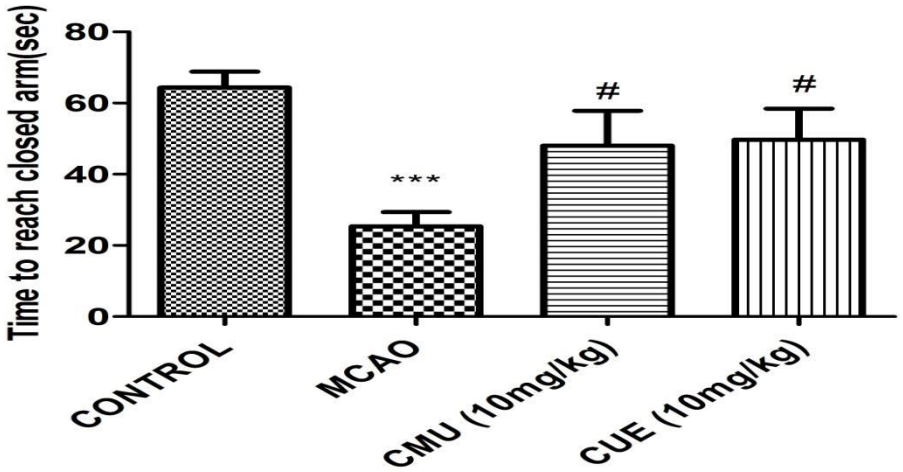
Data are expressed as mean ±SD (n=6).Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. ***denotes statistical significance of MCAo group compared with control group at $p<0.001$. $^{\#,\#}$ denotes statistical significance of CMU (10mg/kg), CUE (10mg/kg) treated groups compared with MCAo group at $p<0.05$, respectively.

FIGURE.19. Effect of drug treatment on time spent in closed arm



Data are expressed as mean ±SD (n=6). *** denotes statistical significance of MCAo group compared with control group at p<0.001. #, ## denotes statistical significance of CMU (10mg/kg), CUE (10mg/kg) treated groups compared with MCAo group at p<0.05, p<0.01 respectively.

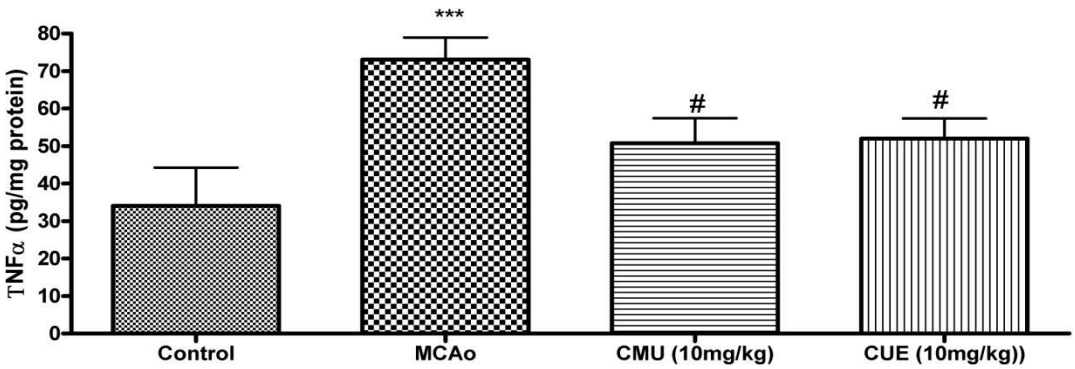
FIGURE.20. Effect of drug treatment on time to reach closed arm



Data are expressed as mean ±SD (n=6). *** denotes statistical significance of MCAo group compared with control group at p<0.001. #, # denotes statistical significance of CMU (10mg/kg), CUE (10mg/kg) treated groups compared with MCAo group at p<0.05, respectively.

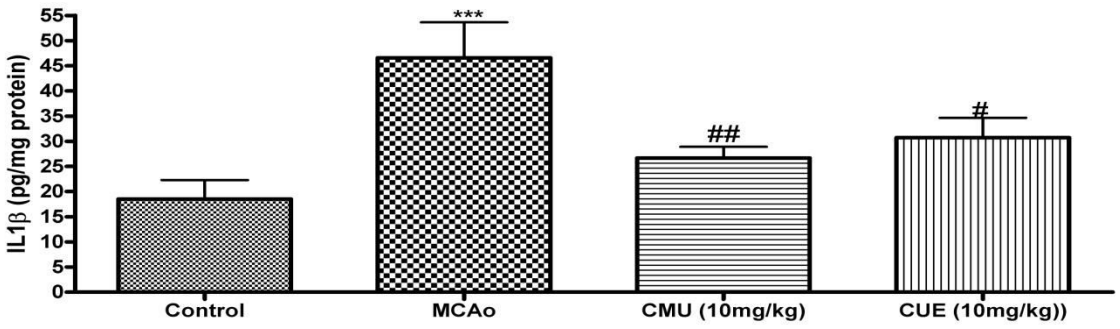
ELISA

FIGURE.21. Effect of drug treatment on pro-inflammatory mediator(TNFα) by ELISA



Data are expressed as mean ±SD (n=6). *** denotes statistical significance of MCAo group compared with control group at p<0.001. #, # denotes statistical significance of CMU (10mg/kg), CUE (10mg/kg) treated groups compared with MCAo group at p<0.05, respectively.

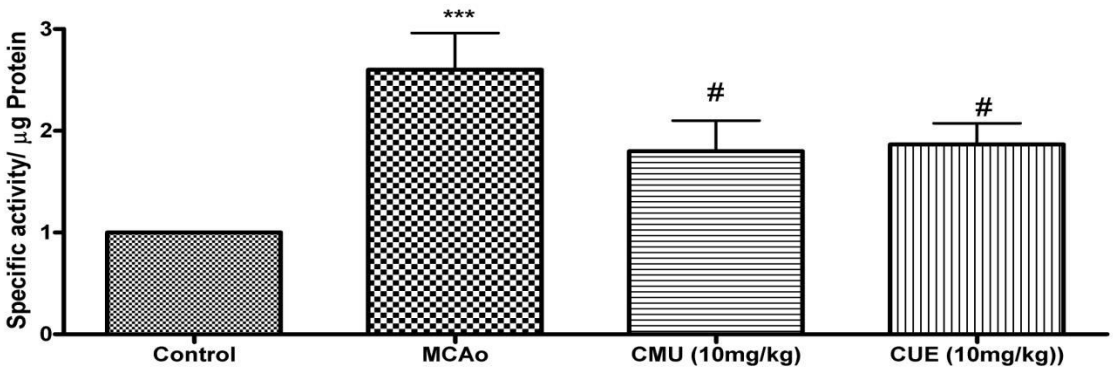
FIGURE.22. Effect of drug treatment on pro-inflammatory mediator(IL 1β) by ELISA



Data are expressed as mean ±SD (n=6).Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. *** denotes statistical significance of MCAo group compared with control group at p<0.001. ##, # denotes statistical significance of CMU (10mg/kg), CUE (10mg/kg) treated groups compared with MCAo group at p<0.01, p<0.05 respectively.

Caspase Activity Assay

FIGURE.23. Effect of drug treatment on caspase activity assay



Data are expressed as mean ±SD (n=6). *** denotes statistical significance of MCAo group compared with control group at p<0.001. #, # denotes statistical significance of CMU (10mg/kg), CUE (10mg/kg) treated groups compared with MCAo group at p<0.05, respectively.

FIGURE.24.A.Effect of drug treatment on BAX & Bcl2 Protein levels

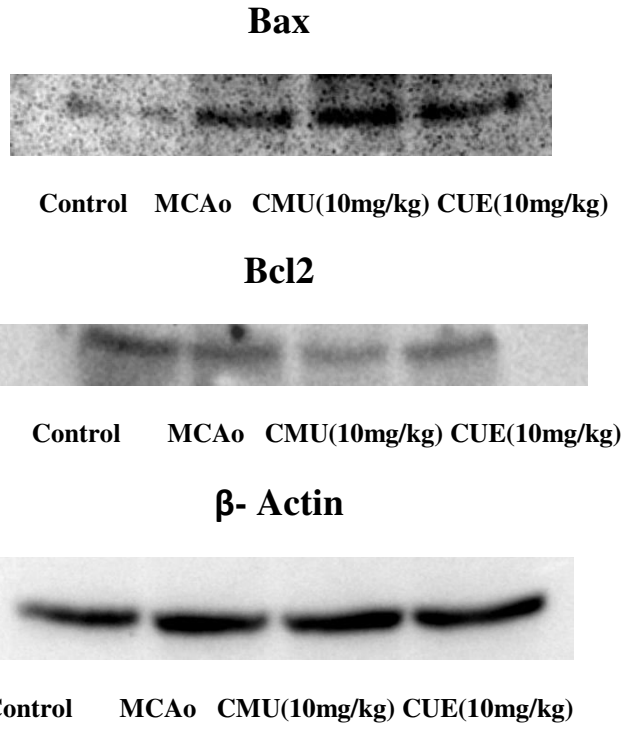
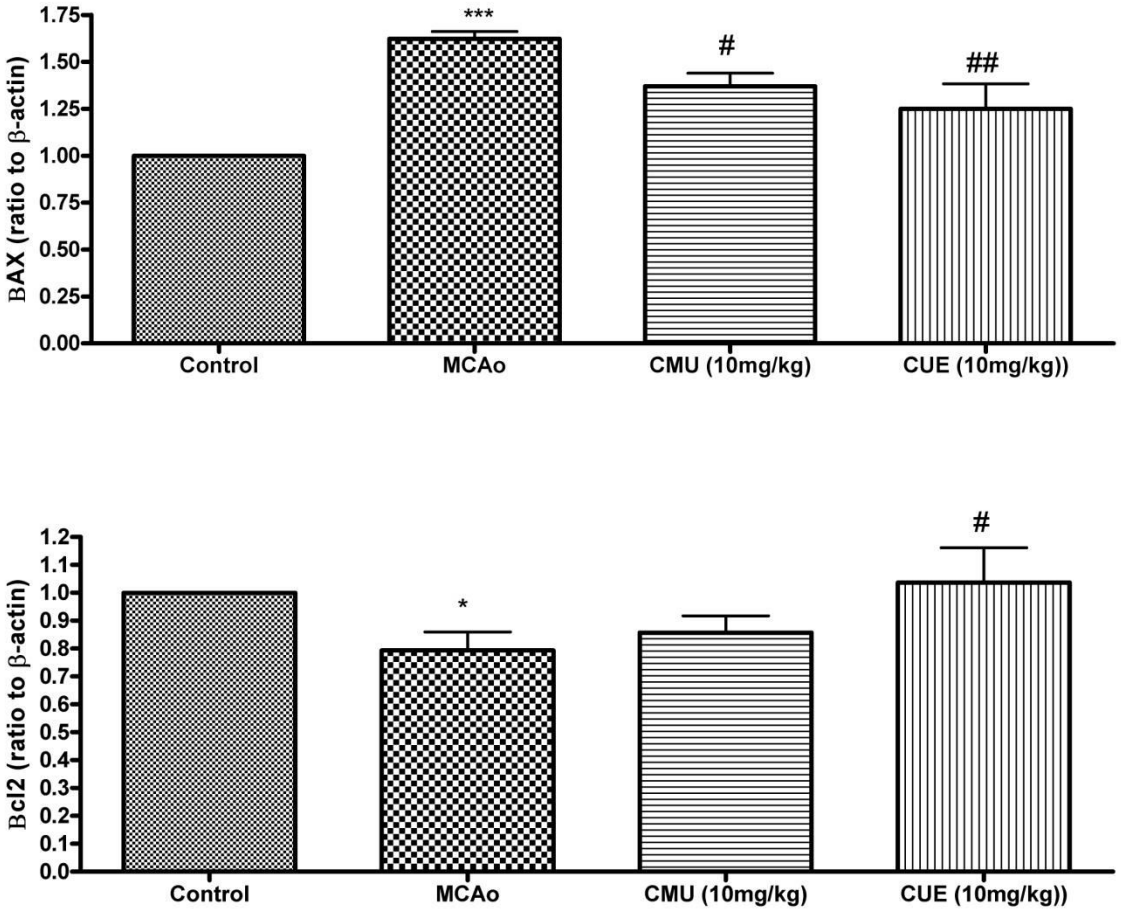


FIGURE 25.B..Effect of drug treatment on Bax & Bcl2 levels



Data are expressed as mean ±SD (n=3). Statistical analysis was carried out using One-way ANOVA followed by Tukey's Multiple Comparison Test. $^{*, ***}$ denotes statistical significance of MCAo group compared with control group at $p<0.05$, $P<0.001$. $^{\#, \#\#}$ denotes statistical significance treated groups compared with MCAo group at $p<0.05$, $p<0.01$ respectively.

Discussion

6.DISCUSSION

Estradiol is thought to exert the majority of its biological actions in the body via interaction with two primary estrogen receptors: estrogen receptor-alpha (ER-α) and estrogen receptor-beta (ER-β) (Deroo *et al* 2006). Both receptors are composed of seven domains, bind E2 with high affinity, and they both dimerize and utilize the classical estrogen response elements in a similar fashion. However, several differences do exist between ER-α and ER-β, as it has been shown that they contain different ligand binding domains, and each receptor is encoded by a different gene. In the brain, localization studies have demonstrated that ER-α is localized most densely in the hypothalamus, hippocampus, and preoptic area, with moderate to light density in the cerebral cortex (McEwen *et al.,* 2001). Conversely, ER-β localization has been documented predominantly in the cortex, throughout the hippocampus, in the olfactory bulb, septum, preoptic area, nucleus of striata terminalis, amygdala, paraventricular hypothalamus, thalamus, ventral tegmental area, substantia nigra and cerebellum (Perez *et al.,* 2003).

While the majority of the literature appears to support a critical role for ER-α in mediating E2 neuroprotective effects against cerebral ischemia, there are studies suggesting that ER-β may have a neuroprotective role in certain situations. For instance, administration of a selective ER-β agonist, WAY 200070-3, has been shown to exert neuroprotection in the rat hippocampal CA1 region following GCI (Miller *et al*.,2005), and another study found that the ER-β agonist, DPN, reduced global cerebral ischemia damage in the mouse hippocampal CA1 region by 55%. In the present study, administration of novel ER-β agonist at dose of 10mg/kg ameliorated the ischemic damage induced by middle cerebral artery occlusion. However, evidence of a role for ER-β in mediating endogenous E2 neuroprotection against cerebral ischemia is currently lacking, as E2 is fully capable of exerting neuroprotection against cerebral ischemia in ER-β knockout mice (Merchenthaler *et al*.,2005). Nevertheless, there is evidence that ER-β may have a role in basal neuronal survival, as it has been reported that there is substantial neuronal loss in the brains of ER-β knockout mice at 2 years of age as compared to wild type mice (Wang *et al*., 2001). Being E2 protective there NCE can be noted on agonist.

Several studies have shown that synthetic SERMs, such as tamoxifen, raloxifene or bazedoxifene and natural SERMs, like genistein (Azcoitia *et al*., 2006), are neuroprotective *in vitro* and *in vivo*. Previous reports suggests that both ERα and ERβ regulated pro-

inflammatory cytokine and chemokine production through E -dependent and E -independent mechanisms (Brown *et al*., 2010). Estradiol and three ERβ-selective compounds, ERB-041, WAY-202196, and WAY-214156, repressed the expression of these inflammatory genes TNF-α, IL-6, and CSF2 by recruiting the coactivator, SRC-2 has been proved. Estrogen receptors and IGF-I receptors cooperate in neuroprotection in animal models of hippocampal neurodegeneration, Parkinson's disease and global cerebral ischemia (Azcoitia *et al*., 1999; Quesada and Micevych, 2004; Garcia-Segura *et al*., 2006; Mendez *et al*., 2006). In addition, IGF-I receptor and estrogen receptor activation synergistically increase the activity of the kinase Akt and coordinately regulate protection from neurotoxicity downstream of Akt which further activates glycogen synthase kinase 3β (GSK3β) (Cardona- Gomez *et al*., 2004). Administration of novel ER-β agonist to MCAo occluded rats did not alter the GSK3β/Akt pathway.

In a recent study that the silencing of hippocampal ER β - increased inflammasome activation; whereas periodic ER- β agonist treatment reduced the activation of the inflammasome, consistent with decreased processing of IL- 1β(de Rivero Vaccari *et al*.,2016). Estrogens can also inhibit expression of pro-inflammatory cytokines such as IL-1β and TNF-α in primary astrocytes following LPS exposure (Lewis *et al*., 2008). In this study, novel ER β agonist down regulated the brain IL-1β and TNF-α level suggesting their role in controlling neuro inflammation in cerebral ischemic condition.

Caspase-3, the final executioner of apoptotic cell death mechanism, is a substrate of calpain and it becomes active following degradation by calpain, and therefore, up regulated calpain is expected to activate caspase-3 in the injured tissue (Gao and Dou.,2000). In our study treatment of novel ER β agonist reduced the caspase 3 activity in MCAo rats.

The selective ERα agonist (propylpyrazole triol, PPT) and ERβ agonist (diarylpropionitrile; DPN) are supposed to show similar neuroprotective actions in culture studies. But a previous report showed higher neuroprotective potential of the ERα agonist PPT than the ERβ agonist DPN (Behl *et al*., 1995). Some other investigations showed that both PPT and DPN can provide neuroprotection against glutamate toxicity by increasing the expression of the anti-apoptotic Bcl-2 protein and also modulating the stress kinase signaling pathways (Zhao et al., 2007). In this study, anti-apoptotic protein bcl2 level were increased and proapoptotic protein levels were reduced on administration of novel ER β agonist to MCAo rats.

Summary & Conclusion

7. SUMMARY & CONCLUSION

- The objective of the study is to asses the behavioural functions of ER-β stimulation in cerebral ischemic condition using novel NCEs.
- To evaluate the mechanism of neuroprotective activity of ER-β agonist in cerebral ischemia by exploring the anti-inflammatory pathways.
- MCAo was induced by occluding middle cerebral artery under anesthetic condtions and the behavaioural parameters were studied after 24 hrs and on 7^{th} day.
- ER-β agonist CMU, CUE (10mg/kg) has been assessed for their neuroprotective action.
- The behavioural parameters include measurement of neurogical deficit through grip strength measurement, anxiety beahviour in open feild and elevated plus maze tests.
- Inducement of MCAo in rats have shown decreased locomtor activity, exploratory, rearing, grooming behaviours, indicates inducement of cerebral ischemia have resulted in anxiogenic behaviour.
- Post ischemic administration of CMU, CUE (10mg/kg) significantly attenuated the behavioural in MCAo rats as observed by increased locomotor activity, exploratory & grooming behavious, grip strength.
- Post ischemic administration of CMU, CUE (10mg/kg) significantly reduced the IL-1β, TNF-α and caspase activity.
- CUE (10mg/kg) significantly reduced the pro apoptotic Bax & anti apoptotic Bcl2 levels.
- Novel ER-β agonist has shown neuroprotective effect by downregulating neuro inflammation and attenuating apoptotic pathway.

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CME Accreditation Certificate

This is to certify that Anandha Kumar. C

has participated in

MOLECULAR BASIS OF MALIGNANCY - CURRENT TRENDS

held at PSG IMS&R on 23-06-2016 as a participant

This activity has been reviewed and accepted by The Centre for Accreditation, The Tamil Nadu Dr. MGR Medical University and the University designates this educational activity for a maximum of 05 Credit points in Category D

Moderator

Dr. S. Ramalingam
Dean

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WORKSHOP ON

ROLE OF PHARMACIST IN CLINICAL RESEARCH

Certificate of Participation

This is to certify that Dr. / Mr. / Ms. Anandhakumar · C

has participated as delegate in one day workshop sponsored by Novartis Healthcare Pvt Ltd, Hyderabad on 24th June 2016 held at PSG College of Pharmacy, Coimbatore

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PSG Institute of Medical Sciences & Research
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DATE: 21.11.2016

Title of the Project: Inflammatory pathway studies in neurodegenerative condition with reference to cerebral ischemia. .

Proposal Number: 345 /2016/ IAEC

Name of the Applicant: Dr.M.Ramanathan

Approval date: 21.11.2016

Expiry date (Termination of the Project): 20.11.2017

Methodology: Approved.

Name of species: **Swiss albino mice/ Wistar rats/ Sprague Dawley rats/ Guinea pigs/ Newzealand White rabbits.**

Male/Female/Both sex----------SD rats 63 male----------animals approved.

Signature of Chairperson

Date: 21.11.16

Dr.M.Ramanathan

Name of the chairperson

The Chair Person, CPCSEA
IAEC of PSGIMS&R
Coimbatore-641 004.

Signature of the CPCSEA nominee

Date: 21/11/2016.

Dr.C.Kathirvelan

Name of IAEC/CPCSEA nominee

Main Nominee, CPCSEA
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